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Highly efficient transfection of *Xanthomonas campestris* by electroporation

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Abstract. *Xanthomonas campestris* pv. *citri* XW47 was transfected with the replicative form DNA of filamentous phage Cf with extremely high efficiency by electroporation. Bacterial cell suspensions treated with Tris buffer containing $MgSO_4$ and NaCl were exposed to high voltage pulses at 12.5 kV/cm for a brief period of time (resistance capacitance time constant = 7-9 msec). Electric transfection of *X. campestris* pv. *citri* results in frequency as high as 5.2×10^9 pfu per μg of DNA. Highly efficient transfection was also obtained with single-stranded DNA of Cf. Other filamentous phage Xf, both RF DNA and single-stranded DNA were efficiently accepted by *X. campestris* pv. *oryzae* 604 under the same condition. Electroporation could be also applied in transferring DNAs of Xf and Cf to non-host *X. campestris* strains. However, the efficiencies of transfection were all lower than the efficiencies for their host strains. DNAs isolated from the phage particles after transfection were identified after hybridization with radiolabeled probe prepared from the single-stranded DNAs of Cf and Xf.

Key words: Bacteriophage; Electroporation; Gene transfer; Transfection; Transformation; *Xanthomonas campestris.*

Introduction

Pathogenic bacteria of plants can be classified into five genus: *Pseudomonas, Xanthomonas, Erwinia, Agrobacterium* and *Corynebacterium*. Among these, *Pseudomonas* and *Xanthomonas* are pathogenic bacteria for most plant species. Since pathogenicity is often determined by the net effect of several genes or groups of genes. Recombinant DNA methodology can be applied to systematically isolate those genes that may contribute to pathogenicity. In order to study the pathogenesis of *Xanthomonas* species on the molecular level, attempts have been made to develop phage vectors and an efficient transformation system for molecular cloning. Electroporation, also known as electropermeabilization, is a kind of electrotransformation which transfers DNA into cells by an high voltage pulse. This technique is applicable to mammalian cells (Potter *et al.*, 1984), plant protoplasts (Shillito *et al.*, 1985) and bacteria (Calvin and Hanawalt, 1988; Fiedler and Wirth, 1988; Luchansky *et al.*, 1988). In *Escherichia coli*, the efficiencies of both transfection and transformation are significantly higher by electroporation than by the $CaCl_2$ method (Dower *et al.*, 1988; Taketo, 1988). The use of electroporation as a method for transforming other bacterial species were also reported (Chassy and Flickinger, 1987; Ito *et al.*, 1988; Luchansky *et al.*, 1988; Miller *et al.*, 1988; Power *et al.*, 1988). Considerable effect had been directed toward developing a genetic transfer system in *Xanthomonas* (Su *et al.*, 1990). However, efficient technique for DNA transformation and transfection have not been described. In this report, we demonstrate the utility of high voltage electroporation as an efficient

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method for genetic transfection of *X. campestris* with bacteriophage DNA. Various parameters affecting the transfection efficiency have been investigated. With the optimal conditions we described, transfection efficiencies higher than 10^{10} pfu per μg of phage DNA have been routinely obtained.

Materials and Methods

Bacterial Strains and Bacteriophages

Xanthomonas campestris pv. *citri* XW47 and *X. campestris* pv. *oryzae* 604, were provided by the Institute of Botany, Academic Sinica, *X. campestris* pv. *campestris* 1701 was supplied by the Agricultural Biotechnology Laboratories, National Chung Hsing University. All bacterial species used were grown in potato-sucrose medium (PS medium), which contained, per liter: potato, 200 g; sucrose, 15 g; peptone, 5 g; $Na_2HPO_4 \cdot 12H_2O$, 2 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g. (Kuo *et al.*, 1967) at 28°C. Filamentous phage Cf, isolated from *X. campestris* pv. *citri*, another filamentous phage Xf and todashaped phage Xp10 isolated from *X. campestris* pv. *oryzae*, were provided by Dr. Kuo, the Institute of Molecular Biology, Academic Sinica.

Preparation of Bacterial Cells

Bacterial cells were grown in PS medium overnight at 28°C. Cells were chilled, sedimented and resuspended in 0.5 volume of TEN buffer (200 mM Tris, pH 7.5; 1 mM EDTA; and 1 M NaCl). After even mixing and centrifugation, pellets were washed and resuspended in TMN buffer (50 mM Tris, pH 7.5; 50 mM $MgSO_4$ and 80 mM NaCl) on ice for 2 h. Pellets from centrifugal separation were then washed and resuspended in deionized water for electroporation.

Electroporation

To determine conditions required for electroporation of *X. campestris* pv. *citri*, RF DNA of bacteriophage Cf was used as a source of transfecting DNA. 1-5 μl (1-2 μg) of DNA in deionized water was added to 40 μl of the bacteria suspension as prepared above. After even mixing, the mixture was poured into a precooled cuvette and subjected to the appropriate pulse. A wide range of field strengths with a fixed capacitance of 25 μF and three different resistances, 100Ω, 200Ω, and 400Ω were applied to the samples. Following the pulse, the cells were removed from the electrodes and mixed with 1 ml of PS medium. The samples were incubated at 28°C for 1 h. Plaque count was determined by means of the double layer method. Transfection efficiency was calculated and expressed in terms of pfu/μg DNA.

Since cellular competence could affect the transformation efficiency, we tested the effect of growth stage on the bacterial transfection by electroporation. Cells of *X. campestris* pv. *citri* growing in PS broth at 28°C were collected at intervals and tested for electroporation.

DNA Purification, Gel Electrophoresis and Southern Blot Hybridization

The DNAs used for transfection were single-stranded DNA (SS) and double-stranded replicative form DNA (RF) of phage Cf and Xf. Double-stranded DNA of phage Xp10 was also used for transfection experiments. Replicative Form (RF) DNAs of phage Cf and Xf were isolated from infected bacterial cells as described by Yang and Kuo (1984) and were purified for use in electroporation by centrifugation to equilibrium in cesium chloride/ethidium bromide density gradient. Phage DNAs were prepared by the method of Earl *et al.* (1987). In preparing a probe, the purified single-stranded DNA of Cf and Xf were labeled with ^{32}P-dCTP by the multiprimer DNA labeling method (Amersham International Plc., England).

To ascertain that the DNA contained in the phage particles after transfection was from bacteriophage Cf or Xf, hybridization was performed with radiolabeled probe prepared from the single-stranded DNA. The DNA was extracted and fractionated on 0.7% agarose gels and transferred to nylon membranes as described by Maniatis *et al*. (1982). Prehybridization for 4 h and hybridization for 24 h at 42°C in formamide solutions were carried out as described. After hybridization, filters were washed three times in 2X SSC-0.1% sodium dodecylsulfate at room temperature for 15 min and three times in 0.1X SSC-0.5% sodium dodecyl sulfate at 68°C for 2 h. Blots were then dried and exposed to X-ray film.

Results and Discussion

Conditions for the Efficient Transfection of ***X. campestris*** *pv.* ***citri***

For field strength of 12.5 kV/cm with a 25 μF

Table 1. *Transfection efficiency of* ***Xanthomonas campestris*** *pv.* ***citri*** *XW47 with Cf RF DNA in different preparations*
Bacterial cells were prepared as described in Materials and Methods. The pretreated cells were mixed with 75 ng/2 μl of Cf RF DNA and subjected to electroporation at 12.5 kV/cm, 25 μF, then diluted and plated with the indicator bacteria for plaque assays.

Pretreatment of bacterial cells	Resistor (Ω)	Time (msec)	Transfection efficiency (pfu/μg DNA)
Water	100	2-3	1.0×10^2
	200	4-5	3.0×10^2
	400	7-9.3	4.0×10^2
TEN and then water	100	2-3	1.0×10^3
	200	4-5	2.0×10^4
	400	7-9.3	5.0×10^4
TEN, TMN and then with water	100	2-3	3.25×10^7
	200	4-5	2.93×10^9
	400	7-9.3	5.27×10^9

capacitor and 200Ω parallel resistor, detectable transfection was observed following the addition of phage DNA to *X. campestris* pv. *citri* XW47. However, a low transfection efficiency of 2.0×10^2 pfu per μg DNA was obtained. Considerable effect was therefore being provided in improving the electroporation efficiency. We found that the pretreatment of samples with salt was important. Preincubation of samples with TEN buffer followed by washing with H_2O resulted in a transfection efficiency of 10^3-10^5 pfu per μg DNA upon electroporation for 4-5 msec at 12.5 kV/cm (Table 1). If TMN buffer was applied after TEN buffer treatment, the efficiency of transfection was reached to 10^7-10^9 pfu per μg DNA. The pretreatment of *X. campestris* pv. *citri* with TMN buffer containing $MgSO_4$ and NaCl therefore had a dramatic effect on the efficiency of electroporation. Since the accumulation of polysaccharide in *Xanthomonas* might contribute to the inhibition of DNA transfer. Treatment of salt with high concentration is required for removing such accumulation and improving the transfection efficiency. Even prolonged incubation in high salt solution, the cells are tolerable to drastic voltage shock. Electroporation may therefore be a practical method for introducing DNA into *X. campestris*.

The compensatory effect of pulse amplitute and duration on electrotransfection are also observed in this study. We have not been successful in obtaining high efficiency transfection with low field strength (up to 7.5 kV/cm). Exponential decay pulses of 10 kV/cm and 9 msec or 12.5 kV/cm and 5 msec can produce high level of transfection (Fig. 1). Our results show that the number of transfectants increases nearly in parallel with electric field strength. However, the upper limit of electroporation apparatus we used is 12.5 kV/cm. We suppose that the transfection efficiency might be higher if a greater electric field strength were available.

The effect of time constant on electroporation efficiency was also investigated. The results in Table 1 demonstrated an increase in the efficiency of transfection as the time constant was varied by adjusting the resistor from 100Ω to 400Ω. The optimal conditions were found to be 400Ω for 7-9.3 msec. Growth stage of bacteria also affected transfection efficiency. The yield of transfectants was optimal with cells in the stationary phase (16 h). Cells in the early to mid-exponential phase were less competent. However, in ordinary transformation technique ($CaCl_2$-treated transformation), cells in the early log phase are usually recommended to prepare the competent cells. In our system, cells in the stationary phases are more competent for electroporation particulary under high resistor or for longer electroporation duration. Calculation of the cellls survived in this situation indicated that no statistically significant lethality was observed. We presume that the importance of high cell concentration in maximizing the transfection efficiency. For purpose of maximal

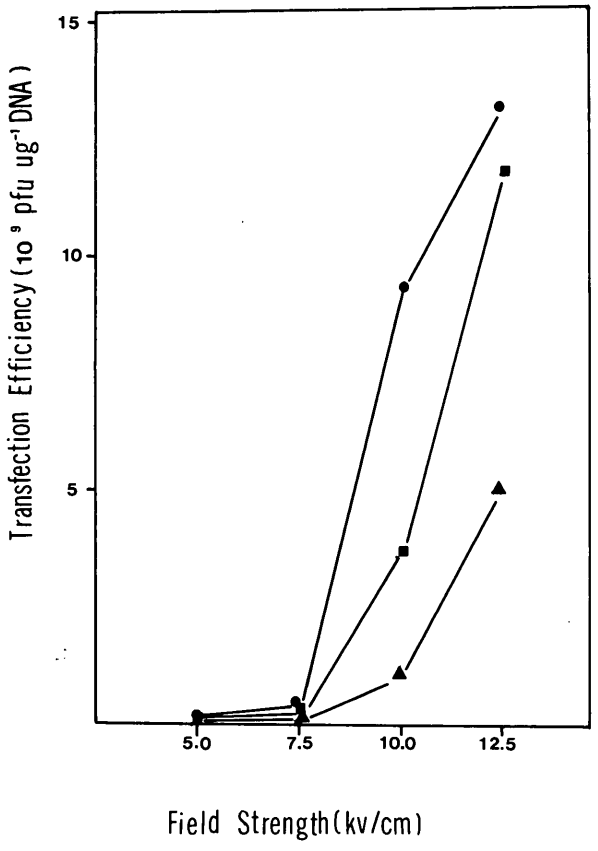

Fig. 1. The effect of electric field strength on the efficiency of transfection. Cells of *Xanthomonas campestris* pv. *citri* XW47 were grown in PS broth at 28°C for 16 hours, washed and suspended in buffer as described in the text. After mixing with 75 ng of Cf DNA, the suspension was subjected to electroporation at various electric field strength with a 25 μF capacitor and 200Ω (▲), 400Ω (■), or 600Ω (●) resistor.

electroporation in *Xanthomonas*, a cell concentration greater than 5×10^{10} cfu/μl is required. In conclusion, growth phase in addition to electric field strength and time constant appeared to have a major effect on transfection efficiency.

Transfection Efficiency in Other ***X. campestris*** *Strains*

Other filamentous phage Xf, isolated from *X. campestris* pv. *oryzae*, both RF and single-stranded DNA were efficiently accepted by *X. campestris* pv. *oryzae* 604 with the efficiencies of 3.1×10^8 pfu per μg DNA and 2.0×10^7 pfu per μg DNA. It suggested that the electroporation conditions used in transfecting *X. campestris* pv. *citri* XW47 may also be applicable to *X. campestris* pv. *oryzae* 604.

Furthermore, DNAs of Xf and Cf could be transferred into non-host *X. campestris* strains by electroporation. Xf can not infect *X. campestris* pv. *citri* XW47, however, its RF and single-stranded DNAs were transferred by electroporation with the efficiency of 3.0×10^4 and 3.4×10^4 pfu per μg DNA respectively. *X. campestris* pv. *oryzae* 604 was not host bacterium of Cf, but could accept RF and single-stranded DNAs of Cf with the transfection efficiency of 1.4×10^5 and 1.9×10^5 pfu per μg DNA. Other *X. campestris* strains such as pv. *campestris* 1701 was also tested for transfection by electroporation. The efficiency of transfection with double-stranded DNA of phage Xf and Cf were 9.6×10^5 and 1.0×10^4 pfu per μg DNA respectively, which are all lower than the efficiency for the host bacteria. This result shows that electroporation can be applied to various *X. campestris* strains, and by this method they could accept both their own phage DNA as well as

Table 2. *Transfection efficiency of different* ***Xanthomonas*** *species with different types of phage DNAs*

Different *Xanthomonas* species were subjected to electroporation with different bacteriophage DNAs under the standard conditions described in Materials and Methods. Transfection efficiency was measured by means of plaque formation.

Bacterial species	Types of DNA	Transfection efficiency (pfu/μg DNA)
X. campestris pv. *citri* XW47	Cf RF	4.3×10^9
	Cf ss	3.3×10^8
	Xf RF	3.0×10^4
	Xf ss	3.4×10^4
	Xp10	0
X. campestris pv. *campestris* 1701	Cf RF	1.0×10^4
	Cf ss	0
	Xf RF	9.6×10^5
	Xf ss	0
	Xp10	0
X. campestris pv. *oryzae* 604	Cf RF	1.4×10^5
	Cf ss	1.7×10^5
	Xf RF	3.1×10^8
	Xf ss	2.0×10^7
	Xp10	5.0×10^5

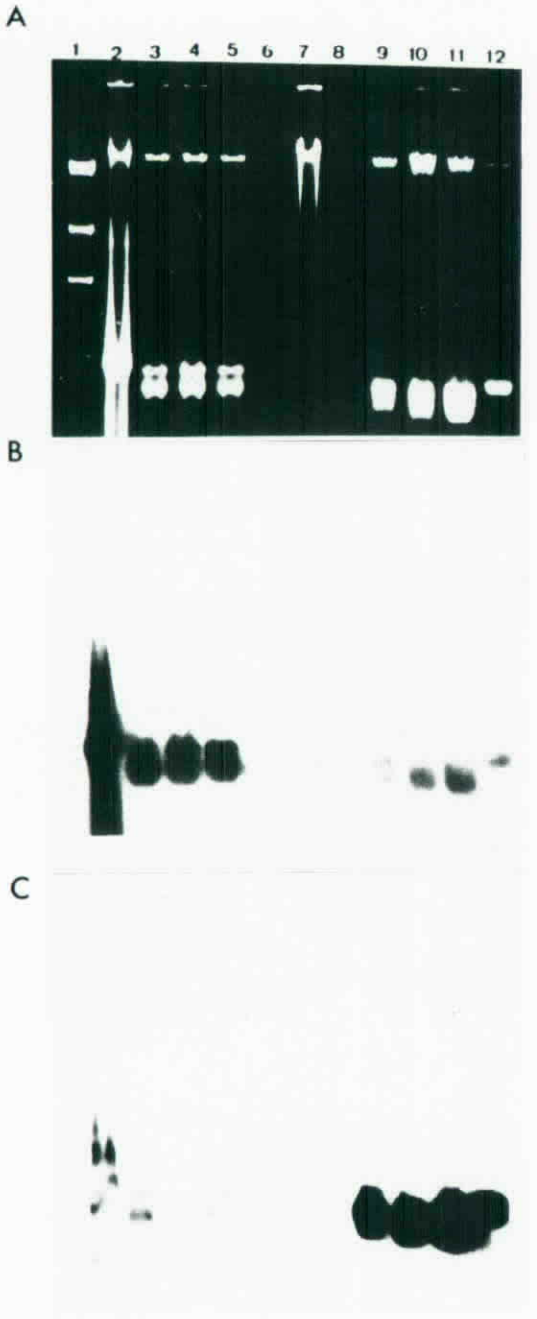

Fig. 2. Southern hybridization analysis of Cf and Xf DNA in various *Xanthomonas* transfectants. (A). Ethidium bromide stain of 0.7% agarose gel of DNA isolated from the transfecting phage particles of *Xanthomonas campestris* strains. (B) and (C). Autoradiogram of the DNA shown in panel (A) after transfer to a nylon membrane and probing with ^{32}P-labeled Cf and Xf ssDNA respectively. Lanes: 1, lambda DNA digested with HindIII; 2, Cf ssDNA; 3, *X. campestris* pv. *citri* XW47 (Cf); 4, *X. campestris* pv. *campestris* 1701 (Cf); 5, *X. campestris* pv. *oryzae* 604 (Cf); 6, *X. campestris* pv. *citri* XW47; 7, *X. campestris* pv. *campestris* 1701; 8, *X. campestris* pv. *oryzae* 604; 9, *X. campestris* pv. *citri* XW47 (Xf); 10, *X. campestris* pv. *campestris* 1701 (Xf); 11, *X. campestris* pv. *oryzae* 604 (Xf); 12, Xf ssDNA.

other phage DNA.

Our observation on the effect of various DNA on electrotransfection indicates that the size rather than the form can affect the transfection efficiency. Single-stranded DNA of filamentous phages Cf and Xf can be transferred to *X. campestris* with similar efficiencies as double-stranded replicative form DNA (Table 2). However, among three *X. campestris* strains, the double stranded DNA of phage Xp10 could only be transferred to the host bacterium *X. campestris* pv. *oryzae* with a transfection efficiency of 5.0×10^5 pfu per μg DNA. This is lower than the efficiency with Xf DNA. We presumed that a unique feature of the host bacterium for phage Xp10 or the larger DNA (46 Kb) limited the transfection of *X. campestris* by electroporation.

Southern Hybridization Analysis of Phage DNA from ***X. campestris*** *Transfectants*

DNA contained in the phage particles after transfection was extracted and analyzed by agarose gel electrophoresis (Fig. 2A). Three *X. campestris* strains contained neither Cf nor Xf DNA. However, DNAs isolated from the phages released by *X. campestris* strains transfected with Cf DNA were hybridized with ^{32}P-labeled probe of Cf (Fig. 2B). It indicated that Cf DNA, after entering the cells, could replicate and produce Cf particles. ^{32}P-labeled probe of Xf DNA also hybridized to DNAs isolated from phages produced by electroporation with Xf DNA in different *X. campestris* cells (Fig. 2C). This result indicated that Xf DNA could replicate in these three *X. campestris* strains and produce their particular phage particles.

Hybridization analysis shown in Fig. 2B and 2C revealed that Xf DNA could react with Cf probe, and Cf DNA could hybridize with Xf probe though there was somewhat a difference in the degree of hybridization. We supposed that the genome of bacteriophage Cf and Xf obviously have a certain degree of identity.

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以電導法進行 *Xanthomonas campestris* 高效率之轉染作用

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線狀噬菌體 Cf 之複製型雙股 DNA (RF DNA),可利用電導法成功地進入柑橘潰瘍病菌 *Xanthomonas campestris* pv. *citri*。細菌懸浮液先以含有 $MgSO_4$ 及 NaCl 之 Tris 緩衝液處理，加入 DNA 後，以 12.5 kV/cm 高電壓作用，可得到 5.2×10^9 pfu/μg DNA 高效率之轉染作用。另一線狀噬菌體 Xf，其 RF DNA 可利用同法進入其寄主細菌 *Xanthomonas campestris* pv. *oryzae* 604，其轉染效率爲 3.1×10^8 pfu/μg DNA。而非線狀噬菌體 Xp10，其雙股 DNA 也可以電導轉染其寄主細菌 *Xanthomonas campestris* pv. *oryzae* 604。至於單股之線狀噬菌體 DNA，與雙股 RF DNA 一樣，可成功地轉染 *Xanthomonas campestris* 菌株。不論 Cf 或 Xf，其 DNA 皆可藉由此電導法轉染 *Xanthomonas campestris* 之非寄主菌株，雖然其轉染效率不若原寄主細菌爲高。由 Cf 轉染菌釋出之噬菌體，其 DNA 可與 Cf DNA 進行雜交反應；由 Xf 轉染菌釋出之噬菌體，其 DNA 可與 Xf DNA 作用，證明以電導進入之噬菌體 DNA 可在非寄主細菌內複製。